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ORIGINAL CONTRIBUTION

Hypovolemic state: age-related influence of water restriction on cardiac nitric oxide synthase in rats

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Abstract
Aim of study We have assessed the influence of water restriction stress on the nitric oxide (NO) synthase in heart and aorta tissues in young 2-month-old and middle-aged 12-month-old rats.
Methods Animals were divided into control and 24- and 72-h water-deprived groups. We evaluated systolic blood pressure (SBP), biochemical parameters, nitrate and nitrite urinary excretion (UNOx), NADPH-diaphorase activity, and protein levels of NOS in the right atria, left ventricle, and thoracic aorta tissues.
Results Water restriction during 72 h increased SBP (16%) in 2-month-old rats but decreased it after 24 and 72 h (9 and 15%, respectively) in 12-month-old rats. Atria, aorta endothelium, and smooth muscle NOS activity increased (32, 63, and 88%, respectively) only after 72 h of water restriction in 2-month-old rats. It also increased not only after 72 h but also after 24 h in atria (27 and 18%, respectively) and in ventricle (39 and 67%, respectively) in 12-month-old rats. Meanwhile, in this group's aorta smooth muscle, the enzyme activity decreased (16 and 7%, respectively). A major difference seen between ages was the changes in UNOx excretion, which decreased in the younger in 24 and 72 h (47 and 81%, respectively) and increased in the middle-aged rats (193 and 389%, respectively). Water restriction did not change cardiovascular endothelial and neuronal NOS protein levels in any group.
Conclusion NO pathways could contribute to the development of age-related cardiovascular adaptation to volume depletion induced by water restriction.

Keywords Heart · Aorta · Nitric oxide synthases · Water restriction · Age

Introduction

Euhydration status is essential for maintaining several physiological processes in healthy humans and animals. The extremes of water metabolism, severe dehydration as well as water intoxication are well-known causes of mortality [1]. It is widely accepted that older individuals are more susceptible to hypohydration after a hypovolemic challenge due to a deficient thirst response and an impaired ability of the kidney to conserve sodium and water. Recent studies have shown that healthy older men do not recover from dehydration as effectively as their younger counterparts [2]. Takamata et al. have reported that older men have a lower ability to recover from acute dehydration associated with reduced osmosensitivity [3]. Although there is no generally accepted definition for the term dehydration, several authors define the terms of mild and severe dehydration as 1–2% and more than 5% loss of body weight, respectively [4, 5]. Different experimental protocols of water restriction were used in order to evaluate the behavioral or physiological adaptation in animals [6, 7].

Since its early description as an endothelial-derived relaxing factor, nitric oxide (NO) has emerged as a fundamental signaling molecule regulating virtually every

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critical cellular function, as well as a potent mediator in a wide range of physiological and pathological conditions. A number of studies have provided evidence that NO may be involved in the regulation of fluid and nutrient homeostasis [8]. NO synthesis activation induced by osmotic stress revealed its implication in water balance and its buffering effect on vasoconstriction and hypertension [9]. It is also worth mentioning that, in another experimental model of acute volume depletion, we demonstrated that hemorrhagic shock induced a time-dependent and specific NO synthase (NOS) activation modulating cardiovascular function [10]. In fact, NO production and its role during hypovolemic state have been mainly focused on the study of the relationship between NO and renal function [11]. However, neither the time required for NO production to be upregulated nor NOS isoform involved has been established up to now in cardiovascular tissues during disorders of water homeostasis. Additionally, it is interesting to note that cardiovascular system is under constant remodeling during aging process [6, 12]. It has been reported that NO synthesis and release normally decrease with the aging process, and this phenomenon may be a result of altered NOS expression and/or increased production of free radical molecules leading to cellular toxicity and impaired second messenger signaling [13, 14].

The intent of this study is to validate the hypothesis that NO pathways may contribute to cardiovascular adaptation to volume depletion induced by water restriction, being this response different with aging. Thus, we examined the effect of water restriction on cardiovascular NOS activity and protein levels in young (2-month-old) and middle-aged (12-month-old) rats. We also evaluated the following: (1) biochemical parameters and hemodynamics and (2) urinary nitrate/nitrite excretion (UNOx) in young adult and middle-aged rats after 24 and 72 h of water restriction.

Materials and methods

Animals

All study protocols were reviewed and approved by the National Administration of Medicine, Food and Medical Technology, Department of Health and Environment of the Nation, Argentina (N° 6344–96). Male Sprague–Dawley rats, obtained through the breeding laboratories of the School of Veterinary (University of Buenos Aires, Argentina), were received at ages of 2 (220–250 g body weight) and 12 months (450–500 g body weight). All animals were housed individually in metabolic cages with an automatic light/dark cycle of 12 h/12 h and fed with standard rat chow from nutriments Purina (Buenos Aires, Argentina) and tap water ad libitum.

Experimental protocols (see Fig. 1 for more details)

Animals of 2- and 12-month-old rats were randomly assigned as follows:

Control group ($n = 15$ each group): rats had continuous access to both food and water.

Water-deprived group ($n = 15$ each group): rats were deprived of water for 24 or 72 h but had continuous access to food.

The animals were placed in the metabolic cage 2 days before the beginning of the experiments in order to adapt to the environment. Systolic blood pressure (SBP) and biochemical parameters as well as the body weight were evaluated after the adaptation period and at the end of the experimental time.

Biochemical parameters

Blood collections were made to determinate serum Na+, hematocrit, plasma osmolarity, and urinary collections for nitrate and nitrite determination. Serum Na+ was measured using ion-selective analyzer. Plasma osmolarity was measured by micro-osmometer (μosmetteTM Micro Osmometer). Hematocrit was determined from duplicate blood-filled hematocrit tubes. Urine volume (mL/min/100 g body weight) was determined gravimetrically. UNOx were measured according to the procedure described by Verdon et al. [15]. Food intake was evaluated in basal condition and after 24 and 72 h of water restriction in 2- and 12-month-old rats in order to assume that the intra-animal variability in UNOx mainly reflected a water restriction–induced change in the rate of endogenous NO production rather than day-to-day differences in exogenous NOx intake.

Hemodynamics parameters

Systolic blood pressure (SBP) was indirectly measured in awake animals by the tail-cuff method using a Grass polygraph (model 79H, Grass Instrument Co., Quincy, MA, USA) [16]. Prior to measuring SBP, rats were warmed in a thermostated and silent room for 40 min. The blood pressure value for each rat was calculated as the average of six separate measurements at each session. Heart rate (HR) was also measured with cardiotachometer triggered by the pulse pressure signals.

At the end of the experimental period, all animals from both groups (control and water-deprived rats) were killed by decapitation to remove right atria, left ventricle, and thoracic aorta segments. In these tissues, Western blot

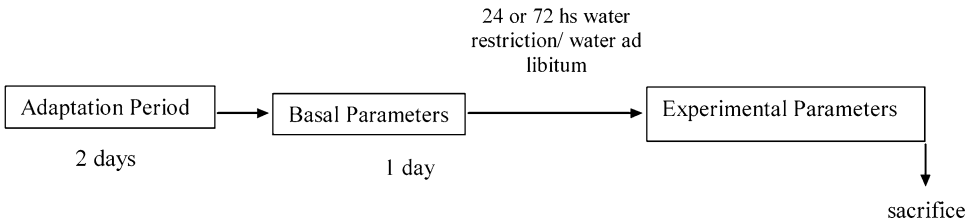

Fig. 1 Experimental protocol

analysis was performed and NOS activity was measured by NADPH-diaphorase (NADPH-d) histochemical method.

Histochemical NOS activity ($n = 5$ each group)

The NADPH-d assay was used for total NOS activity in right atria, left ventricle, and thoracic aorta tissues that were removed at the end point and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were cryoprotected in 15% sucrose and frozen. The sections (15 μm) were cut on a cryostat, mounted on gelatin-coated glass slides, and incubated with a reaction mixture containing 1.2 mM NADPH and 0.24 mM nitro-blue tetrazolium in phosphate buffer added with 0.3% Triton X-100 for 60 min at 37 °C. This technique assays NOS activities that are inhibited by preincubation with diphenyleneiodonium and other NOS inhibitors [17–19]. A Zeiss Axiophot microscope was used for observation, absorption determination, and photography. Computerized image analysis of stained sections was carried out using a Kontron-Zeiss Vidas analyzer, and mean absorption values were calculated from 5 areas of each section and from 10 different sections. The determinations were performed blindly and under similar light, gain, offset, and magnification conditions. No reaction product was found when NADPH was omitted. In control experiments, 5 mM N^G-nitro-L-arginine methyl ester (L-NAME) was added to consider the specific NADPH-diaphorase staining due to NOS activity.

Western-blot analysis ($n = 5$ for each group)

Protein levels of right atria, left ventricle, and thoracic aorta homogenates (0.10 mg protein/lane) were separated by electrophoresis in 7.5% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad, Munich, Germany) and then incubated with rabbit polyclonal anti-NOS antibodies [1:500 dilution: anti-inducible NOS (iNOS), epitope at the COOH terminus; anti-endotelial NOS (eNOS), epitope at the NH2 terminus; and anti-neuronal NOS (nNOS), epitope at the NH2 terminus] and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution). Samples were revealed by chemiluminescence using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology, Uppsala, Sweden) for 2–4 min. Quantification of the bands was performed by digital image analysis using a Hewlett-Packard scanner and Totallab analyzer software (Biodynamics, Seattle, WA). Protein levels were expressed as a ratio of the optical densities of the NOS isoforms and the β-actin bands to control for any inaccuracies in the protein loading. A positive control for each NOS isoform was used to rule out a false-negative result and confirm the antibody specificity. All experiments were performed in triplicate.

Materials

The antibodies against the three isoforms of NOS (iNOS, eNOS, and nNOS), anti-β-actin, were from BD, Biosciences. Western blot detection system and Hybond-ECL membranes were from Amersham Pharmacia Biotech. Biochemicals were from Sigma Chemical Co (Saint Louis, MO).

Statistical analysis

Data in tables, figures, and text are mean values ± SD. For the variables showed in Tables 1 and 2, the data were evaluated with two-way analysis of variance with repeated measures in one factor and the Bonferroni post hoc test for multiple comparisons. For the other variables of the current study, the data were evaluated with two-way analysis of variance and the Bonferroni post-hoc test for multiple comparisons and Kruskal–Wallis and the post hoc test for multiple comparisons when it was appropriated. The Levene's test of equality of error variance was used to evaluate the homogeneity of variances. All statistical procedures were performed using an SPSS statistical software package release 16.0 version. Statistical significance was set at $p < 0.05$.

Table 1 Effects of 24- and 72-h dehydration on SBP, HR, serum Na^+, plasma osmolarity, body weight, and hematocrit in 2- and 12-month-old rats

Time (hours)	Basal		24		72	
Age (months)	2	12	2	12	2	12
SBP (mmHg)	110 ± 2	114 ± 3	113 ± 4	104 ± 3#*	132 ± 9*	100 ± 5#*
HR (bpm)	371 ± 17	382 ± 7	402 ± 10*	424 ± 20#*	480 ± 16*	532 ± 26#*
Serum Na^+ (mmol/L)	136 ± 1	137 ± 2	137 ± 2	140 ± 2	144 ± 2*	148 ± 1*
Plasma osmolarity (mosm)	293 ± 2	294 ± 1	294 ± 1	292 ± 1	323 ± 3*	320 ± 2*
Body weight (g)	281 ± 9	550 ± 24#	270 ± 8*	514 ± 22#*	216 ± 19*	440 ± 21#*
Hematocrit (%)	43 ± 1	44 ± 1	47 ± 3*	50 ± 1#*	47 ± 2*	49 ± 1*

Values are means ± SD, $n = 15$ each group. * $p < 0.05$ versus basal values; # $p < 0.05$ versus respective 2-month-old animals. *SBP* systolic blood pressure, *HR* heart rate

Table 2 Nitrite and nitrate urinary excretion in 2- and 12-month-old rats

NOX urinary (nmol/mL. min. 100 g)	Basal	24 h	72 h
2 months	2.33 ± 0.04	1.23 ± 0.03*	0.44 ± 0.02*
12 months	2.9 ± 0.11#	8.50 ± 0.32*#	14.19 ± 0.38*#

Values are means ± SD, $n = 15$ each group. * $p < 0.001$ versus basal values; # $p < 0.001$ versus respective 2-month-old rats

Results

Biochemical parameters

There were no differences in the basal serum Na^+, plasma osmolarity, and hematocrit between 2- and 12-month-old rats (Table 1). Water restriction for 24 h did not modify serum Na^+ concentration and plasma osmolarity compared with basal values in 2- and 12-month-old rats. This first parameter increased (6 and 8%, respectively) after 72 h of water restriction in both groups of animals. Plasma osmolarity followed a similar pattern of changes to serum Na^+ concentration, increasing (10 and 9%, respectively) in 2- and 12-month-old rats. Water restriction during 24 and 72 h decreased body weight and increased the hematocrit in 2- and 12-month-old rats compared with basal values (Table 1). The hematocrit values of 12-month-old rats after water restriction were greater than those of 2-month-old rats.

Effects of water restriction in baseline systolic blood pressure and heart rate

There were no differences in the basal SBP between 2- and 12-month-old rats. Water restriction during 24 h did not modify SBP compared with basal values in 2-month-old animals. Meanwhile, this osmotic stress during 72 h increased (20%) it compared with basal values in these animals (Table 1). Additionally, Table 1 shows that 24 and 72 h of water restriction decreased SBP (9 and 12%, respectively) compared with basal values in 12-month-old rats. Table 1 also showed that 12-month-old animals had lower SBP after 24 and 72 h of water restriction than their respective young controls. There were no differences in the basal HR between 2- and 12-month-old rats. Meanwhile, water restriction increased it in 2- and 12-month-old rats after 24 h (8 and 11%, respectively) and 72 h (29 and 39%, respectively). Additionally, adult animals had higher HR after 24 and 72 h of water restriction than their respective 2-month-old animals.

Heart tissue

Figure 2 shows that atria (panel A) and ventricle (panel B) histochemical NOS activities were greater in control 12-month-old rats than in 2-month-old animals. Figure 2 (panel A) shows that water restriction for 72 h increased this parameter (32%) in 2-month-old rats. Meanwhile, water restriction for 24 and 72 h increased atria NOS activity in 12-month-old rats (18 and 27%, respectively). Figure 2 (panel B) also shows that water restriction did not change ventricle NOS activity in 2-month-old animals. However, water restriction for 24 and 72 h increased ventricle NOS activity (67 and 39%, respectively) in 12-month-old rats. Western blot analysis revealed that heart homogenates have a positive reactivity against eNOS and nNOS antibody. No reaction with iNOS antibody was observed.

Figure 3 shows that basal atria (panel A) and ventricle (panel B) eNOS expression was similar between 2- and 12-month-old rats. Water restriction did not change eNOS protein levels compared with control rats in neither 2- nor 12-month-old rats. Figure 4 shows that atria (panel A) and ventricle (panel B) nNOS protein levels were greater in 12-month-old rats than in 2-month-old animals. Water restriction did not change atria and ventricle nNOS protein levels, respectively, compared with control rats in neither 2- nor 12-month-old rats.

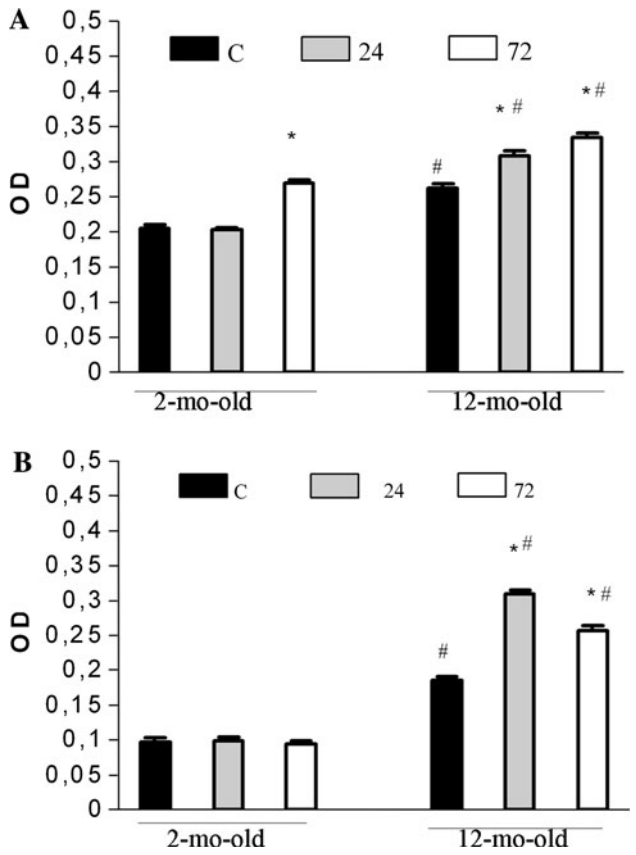

Fig. 2 Histochemical NADPH-diaphorase activity as total NOS activity in the right atria (**a**) and left ventricle (**b**) of 2- and 12-month-old rat hearts. Data are mean ± SD, $n = 15$ rats/group. $^{*}p < 0.05$ respect to C group; $^{\#}p < 0.05$ respect to 2-month-old rats

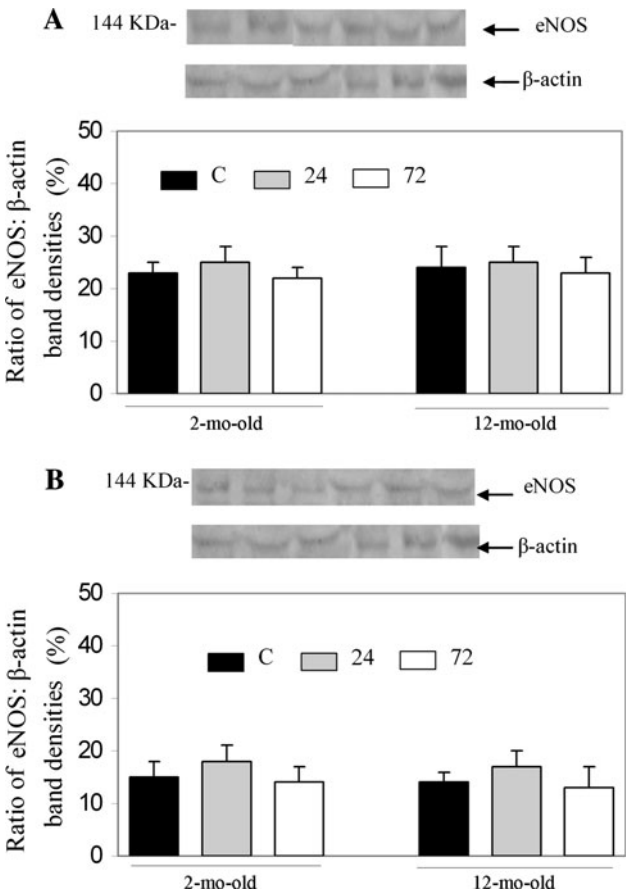

Fig. 3 Representative Western blots of endothelial NOS carried out on proteins from right atria (**a**) and left ventricle (**b**) of 2-month-old rats and 12-month-old rats. Histograms illustrate the ratio between mean endothelial NOS and β-actin protein values for each group. Data are mean ± SD; ($n = 5$ rats/group). $p = NS$

Aorta tissue

Figure 5 shows that endothelium and smooth muscle histochemical NOS activity were greater in 12-month-old than in 2-month-old rats. Aorta endothelium and smooth muscle NOS activity increased (63 and 88%, respectively) after 72 h of water restriction in 2-month-old animals (panel A). However, endothelium aorta NOS activity did not change after water restriction in 12-month-old animals. By contrast, water restriction decreased smooth muscle aorta NOS activity after 24 and 72 h of water restriction in these animals (7 and 16%, respectively) (panel A). Additionally, Fig. 5 shows that aorta eNOS protein levels were greater in 12-month-old than in 2-month-old animals. Water restriction did modify the thoracic aorta eNOS protein levels neither in 2- nor in 12-month-old rats (panel B).

Urinary nitrates and nitrites

Food intake was similar between basal and 24- and 72-h water-deprived 2- (29 ± 1, 25 ± 1, 27 ± 1 g/24 h, respectively) and 12-month-old rats (32 ± 2, 28 ± 1, 26 ± 2 g/24 h, respectively). Urinary nitrite and nitrate excretion was greater in 12-month-old animals than in 2-month-old rats in the time periods studied. Water restriction during 24 and 72 h decreased urinary nitrate excretion in 2-month-old rats (47 and 81%, respectively) compared with basal values. Meanwhile, this urinary nitrate excretion increased in 12-month-old animals (193 and 389%, respectively) compared with basal values (Table 2).

Discussion

The present study was designed to characterize the involvement of NOS in the cardiovascular adaptation to volume depletion caused by water restriction in 2- and 12-month-old rats. Water restriction during 24 and 72 h decreased plasma blood volume as shown indirectly by the increase in hematocrit in 2-month- and 12-month-old rats.

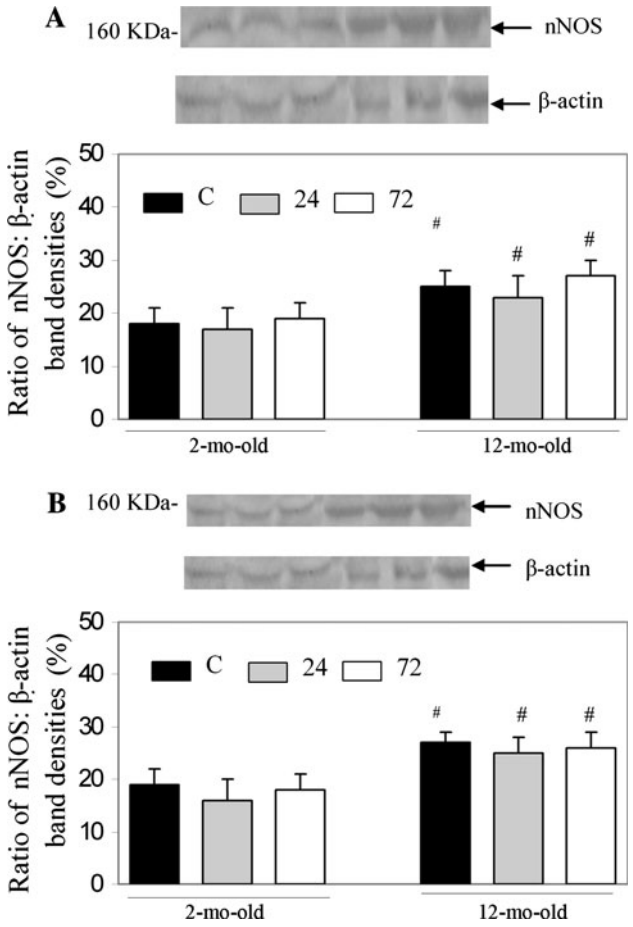

Fig. 4 Representative Western blots of neuronal NOS carried out on proteins from right atria (**a**) and left ventricle (**b**) of 2-month-old rats and 12-month-old rats. Histograms illustrate the ratio between mean endothelial NOS and β-actin protein values for each group. Data are mean ± SD; ($n = 5$ rats/group). $^{\#}p < 0.05$ respect to 2-month-old rats

Additionally, the plasma osmolarity as well as the natremia increased only after 72 h of water restriction in both groups of animals, indicating that a major osmotic stress is needed to alter these parameters, reflecting an extracellular volume decrease. These results imply that the established hypovolemic state depends on the changes in the magnitude of water restriction but it is not influenced by the chronologic age.

Concerning the SBP, it is considerable to note that despite volume depletion after 24 h of water restriction, arterial blood pressure is maintained within the basal range in 2-month-old rats. Meanwhile, this water restriction during 72 h increased it (20%) compared with basal values. However, the impact of water stress on SBP of 12-month-old animals was different. Water restriction for 24 and 72 h decreased it (9 and 15%, respectively) in this group of animals. Several evidences suggests that water restriction increased the peripheral renin–angiotensin system activity, vasopressin circulating levels as well as the sympathoadrenal pathway in water-deprived rats [20–22]. Thus, it is likely that arterial pressure did not fall after 24 h of water restriction in 2-month-old animals, due, in part, to an increase in these homeostatic mechanisms. However, this compensatory response would be altered with advancing age. In response to water stress, aged animals are more sensitive to water restriction, since the threshold for blood pressure regulation is higher compared with 2-month-old rats. However, when we evaluated HR response, our results showed that water restriction increased this parameter in 2- and 12-month-old animals, being this change more influenced by the magnitude of osmotic stress than by the age.

It is also interesting to consider that water restriction not only results in hemodynamic changes but also may induce alterations in cardiovascular NO system. The present study demonstrates that the cardiac NOS activity between control animals is greater in 12-month-old compared with 2-month-old animals. Several studies have provided evidence that in the adulthood, increasing age is accompanied by a progressive decline in the function of cardiovascular system [23, 24]. Thus, it would be probable that the advancing age by itself alters NO production to maintain the chronoctopic response observed in control groups. Moreover, we observed an increase in NOS activity in atria but not in ventricle after 72 h of water restriction in 2-month-old rats. Nevertheless, the cardiac NO bioavailability/production induced by water restriction is different in 12-month-old animals. NOS activity after 24 and 72 h of water restriction was increased in both tissues in this group. In the aging heart, the involvement of NO system would be more necessary since the aged heart might have a degeneration of cardiac sympathetic nerve supply. These findings reflect that probably an impairment of sympathetic tone represents a further factor contributing to age-related cardiovascular response in our experimental condition. However, we cannot throw away the fact that alterations in heart response may be due, at least in part, to changes in kidney adaptation to osmotic stress with advancing age. Additionally, Western blot analysis revealed that the cardiovascular NOS protein levels did not change after water restriction. Thus, the changes in NO production and/or bioavailability may be due to alterations in the regulation of the enzyme activity being independent of modifications of its protein levels. It is important to note that atria and ventricle nNOS protein levels were higher in 12-month-old animals compared with 2-month-old rats. These findings may be explained given that nNOS-derived NO may inhibit sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) function, leading to a contractile dysfunction in the aging heart. Thus, this observation provides evidence that cardiac nNOS isoform is implied in aging process.

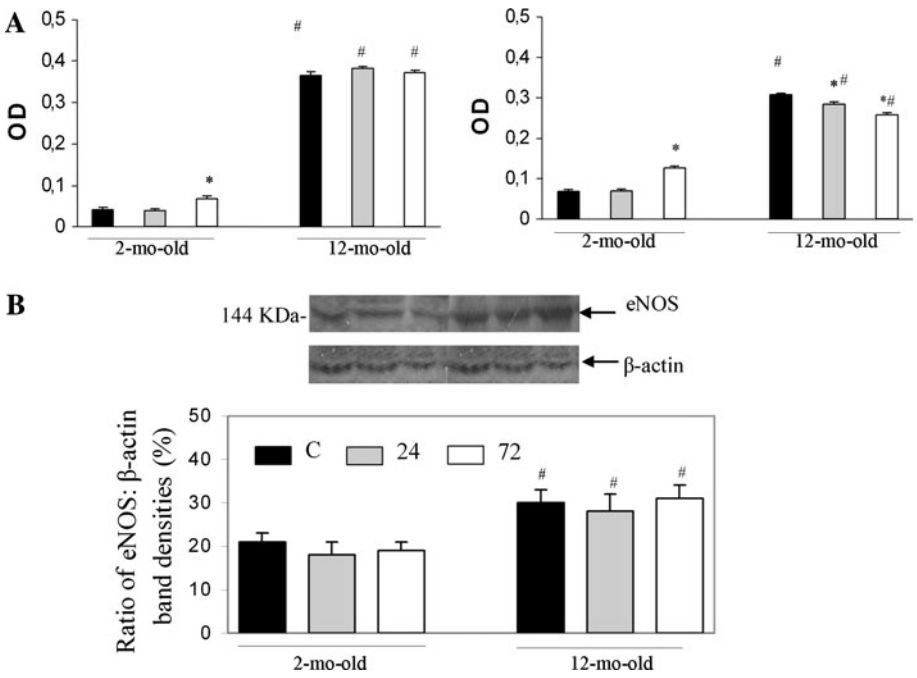

Fig. 5 **a** Histochemical NADPH-diaphorase activity as total NOS activity in the aorta endothelium (*left side*) and smooth muscle (*right side*) of rat in 2-month- and 12-month-old rats. Data are mean ± SD, $n = 15$ rats/group. $^{*}p < 0.05$ respect to C group; $^{\#}p < 0.05$ respect to 2-month-old rats. **b** Representative Western blots of endothelial NOS carried out on proteins from thoracic aorta segments of 2-month- and 12-month-old rats. Histograms illustrate the ratio between mean endothelial NOS and β-actin protein values for each group. Data are mean ± SD; ($n = 5$ rats/group). $^{\#}p < 0.05$ respect to 2-month-old rats

Focusing the physiological role of NO in vascular tissue and being able to evaluate NOS activity in endothelium and smooth muscle, we observed that this parameter is greater in 12-month-old animals compared with 2-month-old rats in control groups. This increased NO-forming mechanism may be due to the advance on age by itself, adjusting to the hemodynamic findings observed in our experimental model. Furthermore, our results showed that histochemical endothelium and smooth muscle aorta NOS activity increased after 72 h of water restriction in 2-month-old rats compared with control group. These findings suggest that changes in NOS activity after 72 h of water restriction are probably triggered by the rise in blood pressure in 2-month-old animals. Meanwhile, 12-month-old rats showed no changes in aorta endothelium NOS activity after water restriction compared with control group. However, this parameter decreased in aorta smooth muscle after 24 and 72 h of water restriction in 12-month-old animals. When we evaluated eNOS protein levels by Western blot analysis, we observed an increase in aged aorta artery compared with 2-month-old animals. Moreover, water restriction did not modify eNOS protein levels in this tissue in both 2- and 12-month-old animals. These findings indicate that 12-month-old animals react less to stressful agents such as water restriction.

On the other hand, we also evaluated the UNOx as an index of endogenous NO production. For instance, differences in exercise level and dietary nitrate and nitrite intake might make UNOx uninterpretable. However, because the dietary intake and physical activity level of all animals were kept under steady and controlled circumstances, we can assume that the intra-animal variability in UNOx mainly reflected a water restriction–induced change in the rate of endogenous NO production rather than day-to-day differences in exogenous NOx intake. Thus, it is likely that the animals whose blood pressure increased during water restriction were unable to increase endogenous NO production and its excretion and that this effect might be, as shown in 2-month-old rats, related to the pressor response observed. The findings after 24 h of water restriction have brought into question the hemodynamic significance of the decrease in UNOx observed after 24 h of the osmotic stress. Thus, there is the possibility that the variation in UNOx, seen in our experimental setting, might not be of hemodynamic significance in 2-month-old rats. Other studies using rats close to 2 months old have shown that water deprivation decreases urine sodium excretion and ANP levels [25], which could alter glomerular filtration rate leading to a reduction in UNOx levels in this group of animals. By contrast, adult animals showed an increase in urinary excretion already after 24 h of dehydration, reflecting that probably an enhanced of endogenous NO production contributed to a reduction in blood pressure after water restriction in these animals.

In summary, we provide evidence to suggest that the cardiovascular impact of a water restriction stress is a predisposing factor for changes in the NOS activity in young 2-month-old and middle-aged 12-month-old rats. Cardiovascular NO pathway adjustments under volume depletion depend on age and adapt in order to support the alterations of the hemodynamic parameters. A main difference seen between ages was the change in nitrite and

nitrate urinary excretion, which decreased in the younger rats and increased in the middle-aged rats, suggesting that NO production depends on the magnitude of the water restriction and on the advance of age.

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